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BIOLOGICAL BULLETIN

THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE DEVELOPMENT OF NORMALLY FERTILIZED *ARBACIA* AND *ASTERIAS* EGGS.

HOMER W. SMITH AND G. H. A. CLOWES.

(From the Lilly Research Laboratory, Indianapolis and the Marine Biological Laboratory, Woods Hole).

The belief that cessation and initiation of development in the marine egg depended in some manner on the ionic equilibria of sea water led Loeb (1) to examine the influence of changes in the concentration of H- and OH-ions on the development of normally fertilized eggs. He found that the development of the eggs of *Arbacia* is retarded and finally prevented if increasing quantities of acid are added to sea water, and that the development to the pluteus stage is accelerated in alkaline sea water. The latter fact was indicated by the advanced size and development of the plutei formed from the treated eggs as compared with controls. On subsequent investigation he concluded that alkali does not accelerate the early cleavage rate, but only the later development from the blastula to the pluteus. The addition of excessive quantities of alkali had an injurious effect. The maximum stimulation was observed when 1.75 cc. *N*/10 NaOH were added to 100 cc. sea water. He attempted to raise the newly fertilized eggs of *Strongylocentrotus* in a neutral Ringer's solution without success, but found that with the addition of a small quantity of KOH, or better $NaHCO_3$, good larvæ might be obtained. He concluded that a neutral or faintly alkaline solution is necessary for normal development (2). This conclusion was reached from other points of view by Herbst (3) and Peter (4).

Moore, Roaf and Whitley (5) performed similar experiments with the eggs of *Echinus esculentus;* the addition of small amounts

of alkalies or alkaline salts, such as Na_2HPO_4, to sea water in which the eggs were growing caused an increase in the rate of growth in the early as well as the late stages, but larger amounts led to abnormal division. They pointed out that in some eggs in quite alkaline solutions nuclear division occurred without cytoplasmic division, so that the blastomeres became multi-nucleated. Still larger amounts of alkali inhibited both nuclear and cytoplasmic division. On the other hand, the smallest amount of acid had only an inhibitive action. There was no tendency for nuclear division without cytoplasmic division; and with comparatively small amounts of acid cell division was completely prevented. They concluded that the extreme limits of reaction at which cell division is possible lie very close together, and they pointed out that the phosphates and carbonates in sea water have a "steadying action" against fluctuations in the concentrations of H- and OH-ions which must be advantageous to cell growth. Subsequently, Whitley (6) found that small quantities of acid and alkali were very injurious to the developing eggs of the plaice, *Pleuronectes platessa*. No accelerating effect was observed in alkaline sea water, but Whitley concluded that a disturbance of the equilibrium towards the acid side is much more fatal than the opposite. There appeared to be an increase in resistance to unfavorable reactions developed in proportion to the age of the larvæ.

Glaser (7) repeated Loeb's experiments with *Arbacia* in another connection and concluded that accelerated development in alkaline sea water is limited to the development from the blastula to the pluteus, and that the early rate of cleavage is not accelerated, and may even be suppressed. Glaser noted the time required for the successive cleavage planes to appear in the majority of eggs in the cultures. By this method a small change in velocity of development would be difficult to detect, though it would become manifest if continued to the later stages where its results would of course be magnified.

Although it had another objective, the excellent work of Medes (8) on the causes of variation in the larvæ of *Arbacia* is of interest in this connection. Medes made careful comparative measurements on the skeletons of plutei obtained by inseminating and rais-

ing the eggs in sea water to which various substances had been added. She found that HCl, CO_2 and acetic acid markedly retarded development. In view of Loeb's statement that alkali does not have any effect on the early development of *Arbacia*, she reexamined this point by counting the number of divided eggs one hour after insemination, and by comparing the skeletal development of the larvæ 18 hours after insemination. She found a definite acceleration from one to 18 hours with NaOH (greatest in 1.33 cc. $N/10$ NaOH + 98.66 cc. sea water) and with Na_2CO_3 (greatest in 0.4 cc. 0.45 M Na_2CO_3 + 99.6 cc. sea water), though in later stages the alkali cultures showed a retardation so that ultimately they lagged behind the controls. Larger quantities of NaOH and Na_2CO_3 inhibited development from the beginning. NaCl produced slender, perforated skeletons with conspicuous processes; there was inhibition during early development and excessive growth during later periods. NaOH led to irregularity and asymmetry, while $NaHCO_3$ increased the bulk of the skeleton with a strong tendency for regularity and symmetry.

Richards (9) has recently observed acceleration of the early cleavage rate of the eggs of the opisthobranch, *Haminea virescens*, in sea water to which NaOH and KOH had been added. No acceleration was observed after the addition of $Ba(OH)_2$ and $Cr(OH)_3$.

In none of the investigations cited were the H-ion concentrations determined or controlled, nor was allowance made for any specific influence which the carbonic acid present in acidified sea water might have. Knowing that carbonic acid inhibits cell division at H-ion concentrations which otherwise are innocuous (10), it is important to determine the limits of reaction of CO_2-free sea water within which normal development is possible.

In performing the experiments reported in this paper, *Arbacia* and *Asterias* eggs were inseminated in sea water and subsequently transferred to 100 cc. of the pH solutions prepared as described in a previous paper (11). At appropriate intervals samples of 3 to 5 cc. were removed from each lot and fixed by the addition, in the case of Arbacia, of 2 or 3 cc. of a 1–1000 solution of formalin in sea water; the *Asterias* eggs were fixed by adding 2 or 3 cc. of a 1–1000 mercury bichloride solution in sea water. These meth-

ods of fixation stop all developmental processes at once and the cleavage planes remain clearly visible for many hours. Careful counts were then made on each sample, noting the number of eggs which were undivided and the number which were in each stage of division. By multiplying the number of two-cell eggs by one, the 4-cell eggs by two, the 8-cell eggs by 3, etc., and dividing the total number of divisions by the total number of eggs, the number of divisions per egg in each sample was determined. This figure is an arithmetic index of the degree of development, or if expressed in terms of time, of the velocity of development. By counting two to 3 hundred eggs in each sample, considerable accuracy can be obtained.

A particular culture of eggs will develop under constant conditions in sea water with a mean velocity that remains practically constant so long as the number of cleavages can be accurately counted. Certain individuals will be slower than the mean and others will be faster than the mean, expressing differences in viability or developmental capacity. Such differences may be interpreted from a statistical point of view to indicate the fluctuations which any individual may undergo, and the mean to represent the behavior of the average individual. The variations observed in the development of different cultures present many interesting features which we cannot discuss at this time. It should be pointed out, however, that for studies of developmental velocity under normal and abnormal conditions, the ideal condition is to have a maximum distribution of variants ("slow" eggs and "fast" eggs) so that development will progress over short intervals of time (*i.e.*, 15 to 20 minutes) in a uniform manner. Though this condition usually obtains, there are times in the season when the eggs are in such uniform physiological condition that they divide almost simultaneously. At such times the number of divisions per egg increases by abrupt steps. This circumstance can be alleviated by averaging two successive observations on each culture. For the present purposes it will suffice to consider the mean development during the entire period of observation.

The influence of reaction on the early developmental rate of normally fertilized *Asterias* and *Arbacia* eggs is shown in Fig. 1. The data summarized in this figure are taken from several experi-

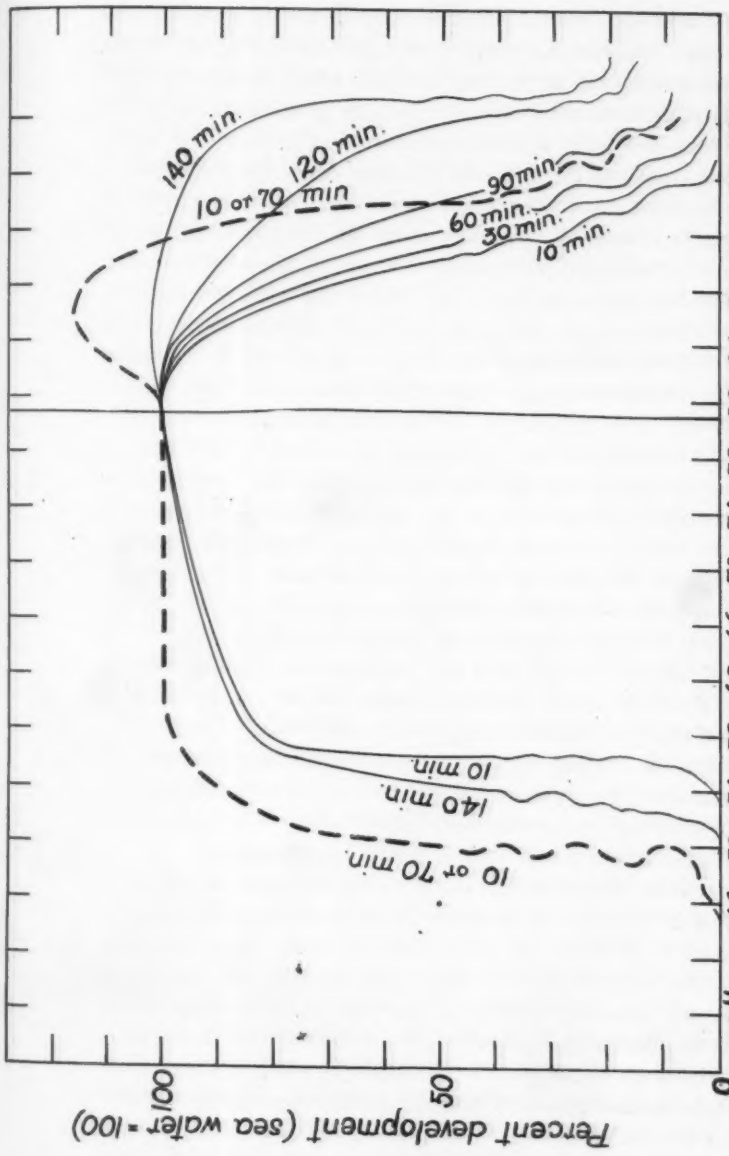

FIG. 1. The influence of H-ion concentration on the early development of normally fertilized *Arbacia* (dotted) and *Asterias* (solid line) eggs. The curves are based on the average results obtained from several experiments with each species and are expressed on the basis of the development in sea water (pH 8.15) as 100 per cent. The figures by each curve show the time after insemination at which the eggs were transferred from sea water to the acid and alkaline solutions.

ments with each species performed during the summers of 1922 and 1923. In these experiments the eggs were inseminated in sea water, centrifuged at various intervals after insemination and transferred to the pH solutions. The data given beside the curves show the time after insemination at which the eggs were transferred from sea water to the pH solutions. The development was followed quantitatively on samples taken every 20 minutes in which the number of divisions per egg was determined by careful counts. The mean development was then obtained by averaging all the observations for each solution, and the results expressed in terms of the corresponding figure for sea water as 100 per cent. The full ordinate indicates the H-ion concentration of sea water.

The dotted line in Fig. 1 refers to *Arbacia* and the solid lines to *Asterias*. The significance of the wavy portions of these lines will be discussed later. In *Arbacia* the velocity of division remains practically constant from pH 8.15 (the H-ion concentration of sea water) to pH 6.0; at 5.0 velocity of division is reduced by one half and at 4.6 division is completely suppressed. A slight increase in the alkalinity of sea water increases the velocity of division; this stimulation reaches its maximum about pH 8.8, and amounts to a 15 to 25 per cent. increase over the velocity in sea water. Above pH 8.8 there is an abrupt retardation so that the developmental velocity is reduced by one half at 9.6, and at 10.12 only a small fraction of the eggs divide even once.

Attention is called to the fact that the limiting reactions are characterized, not by a gradual, but by an abrupt inhibition of cell division within a comparatively narrow range. Between these limiting reactions cell division is essentially unimpaired.

It will be convenient for purposes of reference to define the critical limit as the pH at which the curve under consideration is reduced to its midpoint, *i.e.*, to 50 per cent. Accordingly the limits for *Arbacia* may be said to be pH 5.0 and 9.6. These limits are the same whether the eggs are placed in the pH solutions 10 minutes or 60 minutes after insemination. In the latter case, however, the degree of stimulation by alkali is slightly less.

The *Asterias* egg differs from the *Arbacia* egg notably in this—that while the resistance of the latter to both acid and alkali appears to be the same 10 minutes and 60 minutes after fertilization,

the resistance of the *Asterias* egg is much lower 10 minutes after fertilization than it is later on. There is a gradual increase in resistance, particularly in alkali, from 10 minutes after fertilization until the first cleavage, at which time the maximum resistance appears to be reached. Reference to Figure 1 will show that development is inhibited at pH 9.2 if the eggs are transferred to this solution 10 minutes after fertilization; but if the eggs are not transferred until the majority have reached the two cell stage (*i.e.*, about 140 minutes) they not only tolerate pH 9.2 but they tolerate equally well pH 10.0. This difference in resistance is strikingly shown by the cultures 24 hours later. When placed, for example, in pH 9.2 10 minutes after fertilization the velocity of development is greatly reduced; in many eggs the nucleus divides without cytoplasmic division; fragmentation and abortive division are predominant and no egg progresses beyond the 32-cell stage. When placed in this same solution after the first cleavage has occurred 90 per cent. of the eggs will develop to practically normal swimmers. The increase in resistance to acid is considerably less, the limiting acidity shortly after fertilization being pH 5.6, and 140 minutes after fertilization pH 5.4. The increase in resistance to alkali which gradually appears as the egg approaches the first cleavage plane is not to be confused with the period of great susceptibility which follows the event of fertilization, or with the periodic changes in resistance to various destructive agents which a number of observers have shown to be associated with the process of cleavage. The shortest interval after fertilization at which we transferred the eggs to the pH solutions was 10 minutes, and therefore the period of great susceptibility to destructive agents immediately following fertilization was avoided. And since in our experiments the eggs are left in the pH solutions until the conclusion of the experiments, which cover in the case of *Asterias* 5 cleavages, any periodic fluctuations in resistance, if they occur, are translated into a mean.

There is little increase in the velocity of division in alkaline solution, 2 or 3 per cent. being the maximum observed in any of our experiments. There is frequently a marked stimulation shortly after transferring to the alkaline solution, but this is transient and is followed by a decrease in the velocity of division, so

that after 4 or 5 cleavages the alkali cultures are about even with the controls. There is a slight but perceptible decrease of the velocity of division in acid solutions between pH 5.8 and 7.8, in contrast to the *Arbacia* egg where the velocity remains practically constant.

If we consider the limits for eggs transferred to the pH solutions at the time of the first cleavage, these limits are pH 5.4 and 10.1. Thus the limits within which the development of *Asterias* eggs is possible are distinctly on the alkaline side of those for *Arbacia* eggs.

In those solutions in which the velocity of development is reduced below 50 per cent., the quality of cell division in both *Arbacia* and *Asterias* eggs is greatly altered. The division of the cytoplasm is apparently restrained before the division of the nucleus, and in consequence the majority of eggs become multinuclear. This condition of abnormal division is indicated in Fig. 1 by the wavy portions of the curves. After two or 3 cleavages of the nucleus without cytoplasmic division the egg usually divides abruptly into more than two blastomeres, but the division is invariably abnormal and either soon ceases entirely or leads to cytolysis. In some cases it can be observed that the cytoplasm begins to divide but the furrow melts and the blastomeres fuse. The tendency for nuclear division without cytoplasmic division is much more marked in alkaline than in acid solutions. A point is reached on the alkaline side, however, where nuclear as well as cytoplasmic division is completely inhibited. A similar repression of cytoplasmic division without complete repression of nuclear division has been observed with lack of oxygen, the action of chloroform and ether, the action of hypertonic and hypotonic sea water, cold and other agents (12).

We are concerned here principally with variations in developmental velocity which are made manifest in the early history of the dividing egg, during that period of time in which accurate quantitative information can be obtained. It is of interest to consider, however, the effects of longer exposures. A method is not available for expressing these effects quantitatively but a fair idea of the degree of retardation during a 24 hour exposure can be obtained by comparing the general development of the larvæ.

Such comparisons have shown that some retardation of development occurs even at pH 7.6 and 8.5, and that the effect of increasing acidity or alkalinity does not take the form of an abrupt inhibition at any point, but manifests itself in almost imperceptible gradations from normal development to no development at all. In the acid solutions the inhibition culminates in coagulation with little division; and in alkaline solutions in either complete cytolysis or in the formation of formless, ciliated masses of protoplasm swimming within the fertilization membrane. It is doubtful if normal development can be obtained throughout a period of 24 hours in solutions more acid than pH 7.8, or more alkaline than 8.4.

Summary.

The effect of acid and alkaline sea water on the rate of cell division in normally fertilized *Arbacia* and *Asterias* eggs was observed as far as the 128-cell stage.

In *Arbacia*, the velocity of division is reduced to 50 per cent. of the velocity in sea water (pH 8.15) at pH 5.2 and 9.4. Between pH 5.8 and 8.2 these eggs divide normally both in respect to velocity and quality of cell division. Between pH 8.2 and 9.2 the velocity of division is increased from 15 to 25 per cent.

Asterias eggs are more sensitive to both acid and alkaline sea water during the precleavage period than at any subsequent time. When these eggs are transferred to the acid and alkaline sea water immediately after fertilization, the velocity of division is reduced to 50 per cent. at pH 5.6 and 9.2; when transferred in the two cell stage the corresponding limits are pH 5.4 and 10.2. There is a slight decrease in the mean velocity of division between pH 8.2 and 5.8, but no significant increase in solutions more alkaline than sea water.

In both species, when the developmental velocity is reduced below 50 per cent. by either acid or alkali, the nucleus tends to divide without division of the cytoplasm, and abnormal multinuclear cells are formed.

We are indebted to Mabel T. Studebaker for the statistical work in the experiments recorded in this paper.

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THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE FERTILIZATION PROCESS IN *ARBACIA, ASTERIAS* AND *CHÆTOPTERUS* EGGS.

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In a previous communication we pointed out that when *Asterias* and *Arbacia* eggs were inseminated in CO_2-free sea water of varying H-ion concentration, fertilization failed to occur in solutions more acid than pH 6.6 to 7.0. This block to fertilization appeared to be perfectly reversible, since eggs which did not fertilize in solutions on the acid side of the block could be fertilized when returned to solutions of greater alkalinity (1). Loeb (2) has observed a similar block to fertilization in artificial salt solutions. He found that *Arbacia* and *S. purpuratus* eggs were not fertilized in a neutral mixture of $NaCl + MgCl_2$ in the proportion in which these salts exist in sea water. These eggs were fertilized, however, if NaOH, NH_4OH, benzylamine, butylamine or $NaHCO_3$ were added to the $NaCl + MgCl_2$ mixture. The addition of $CaCl_2$ to the $NaCl + MgCl_2$ mixture similarly made fertilization possible. The addition of NaOH or $CaCl_2$ to a $NaCl + KCl$ mixture did not permit fertilization of all eggs, but when both NaOH and $CaCl_2$ were added to a $NaCl + KCl$ mixture as a rule all the eggs fertilized and began to divide. Since cross fertilization can be effected between *Asterias* sperm and *S. purpuratus* eggs by the addition of NaOH or $CaCl_2$ to normal sea water, Loeb concluded that the act of diminishing the alkalinity of the solution or of depriving it of $CaCl_2$ established the same reversible block to the entrance of the homologous sperm as exists for the entrance of the sperm of *Asterias* into *S. purpuratus* eggs in normal sea water. Leob's experiments involve the change of several variables at once, however, and it cannot be determined from them to what extent the reaction of the external medium

per se influences the fertilization of eggs by sperm of the same species.

Further examination of the block to fertilization which is created when the H-ion concentration of sea water is increased to a critical point has convinced us of its physiological significance, and we have extended our observations to include the effects of increasing alkalinity on the fertilization of *Arbacia* and *Asterias* eggs, and the effects of acid and alkaline sea water on the fertilization of the eggs of *Chætopterus pergamentaceous*.

The Acid Block to Fertilization.

CO_2-free sea water solutions were prepared as described in a previous paper (3). Our experiments on fertilization were performed as follows: A drop of concentrated egg suspension was added to 50 or 100 cc. of each of the pH solutions, and a drop of sperm suspension was added to about 5 cc. of the pH solutions. After an interval of 3 to 5 minutes the sperm and eggs were mixed and thoroughly agitated. (No precautions were taken to remove body fluids which might be present around the eggs, other than the routine washing which they were always given in preparing them for any experiment.) Subsequently the proportion of fertilized eggs in each dish was carefully determined. It makes no difference whether the counts are made 10 minutes or several hours after insemination because every egg that is going to fertilize will lift a membrane within the normal time of 3 to 5 minutes. It has been our custom in performing experiments of this kind to remove samples from the pH solutions 10 or 15 minutes after insemination and return them to sea water with fresh sperm to make sure that the eggs had not been irreversibly modified by the action of the pH solutions or by contact with sperm in these solutions. It may be said that this procedure has one invariable result; if the exposure is below that required for the acid to injure the egg, then every egg which is not fertilized on the acid side of the block will fertilize when returned to sea water with fresh sperm.

The influence of H-ion concentration on the fertilization of *Arbacia* and *Asterias* is illustrated in Figs. 1 and 2. The solid line in each figure indicates the range within which fertilization

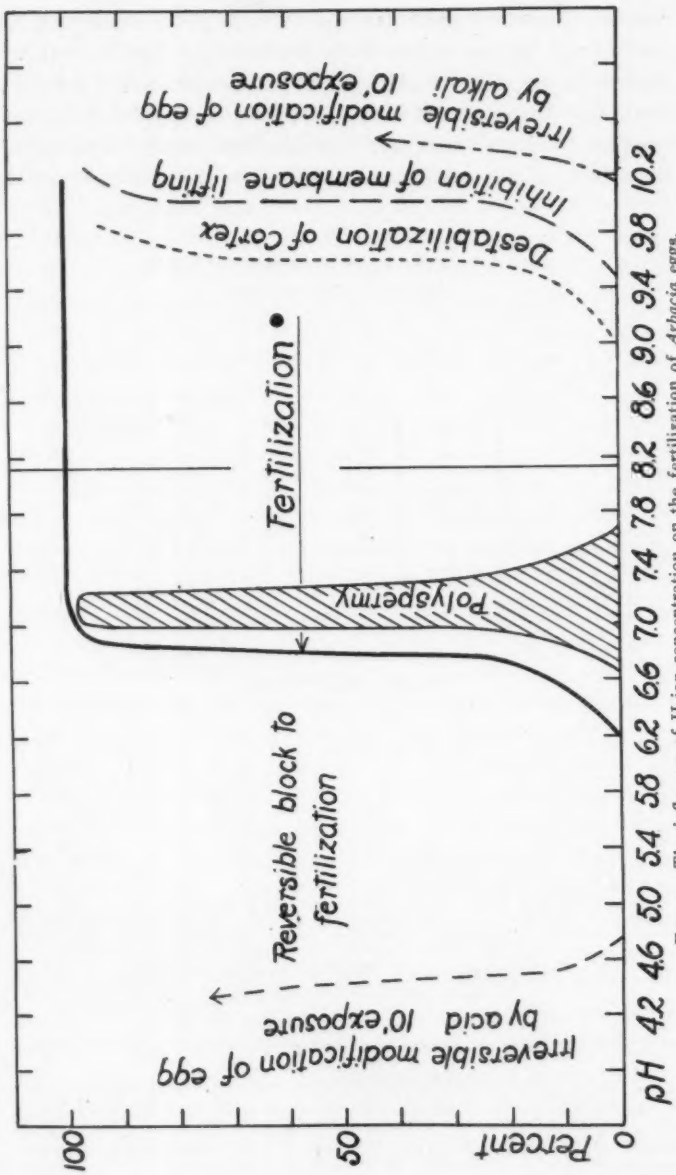
Irreversible modification of egg by alkali 10' exposure
Inhibition of membrane lifting
Destabilization of Cortex
Fertilization
Polyspermy
Reversible block to fertilization
Irreversible modification of egg by acid 10' exposure
pH 4.2 4.6 5.0 5.4 5.8 6.2 6.6 7.0 7.4 7.8 8.2 8.6 9.0 9.4 9.8 10.2
Percent
0
50
100

FIG. 1. The influence of H-ion concentration on the fertilization of *Arbacia* eggs.

occurs. The cessation of fertilization with increasing acidity is very abrupt, but the critical H-ion concentration may be most accurately indicated by the pH at which only 50 per cent. of the eggs fertilize. This critical H-ion concentration is pH 6.8 for *Arbacia* eggs, and 7.0 for *Asterias* eggs. In solutions slightly more alkaline than these, all the eggs fertilize; and in solutions slightly more

TABLE I.

A. ACID BLOCK TO FERTILIZATION IN *Asterias*.

Per Cent. of Eggs Fertilized.

pH									Date.
6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4	
0		0		50		100		100	Conclusions 1921.
0		1		42		100			6–30–22
0		0		21		85			7– 6–22
0		0		0		4		76	5–29–23
0		0	1	5	5	40		72	5–30–23
0	0	0	0	47	60	85		93	8–31–23
0	0	0	0	0		82		100	8–31–23
0	0	0	0	60		100		100	8–31–23
0	0	0	10	6		21		72	9– 2–23
0	0	0	14	11		22		90	9– 3–23
0	0	0	40	40		100		100	9– 3–23

B. ACID BLOCK TO FERTILIZATION IN *Arbacia*.

Per Cent. of Eggs Fertilized.

pH									Date.
6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4	
0		50		100		100		100	Results of 1921.
2		47		98		98		97	6–30–22
0	0	100	100	100	100	100		100	7–20–23
0	0	30	20	75	100				9–21–23
2	7	28	24	95	100	100	100		9–22–23
7	29	100	100	100	100	100	100	100	9–22–23
0	0	30	20	75	100	100	100	100	9–22–23

C. COMPARISON OF ACID BLOCK IN *Asterias* AND *Arbacia* IN SAME pH SOLUTIONS. 9–21–23.

Per Cent. of Eggs Fertilized.

	pH								
	6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4
Asterias	0	0	0	1	30	100	100	100	100
Arbacia	5	0	30	70	100	100	100	100	100

acid, none of the eggs fertilize. We have previously shown that the unfertilized eggs of these species are uninjured by short exposures to the solutions in which fertilization does not occur (3); and that normally fertilized eggs will develop with normal velocity at H-ion concentrations much greater than those at which the block to fertilization occurs (4).

The constancy of this acid block to fertilization is very marked. To illustrate this a few experiments have been given below. Table I. contains a résumé of experiments performed in 1921, 1922 and 1923 on the fertilization of *Asterias* and *Arbacia* eggs. Despite the probability of variable conditions in these experiments, the point at which 50 per cent. of the eggs fertilized remained constant to $\pm$ 0.2 pH.

There are at least two factors which might be expected to shift the block one direction or another; first, the length of time which the eggs or sperm have remained in the acid solution, and second, the relative quantity of sperm used for insemination. Examination of the first factor has shown that the equilibrium between the pH solution and the egg (or sperm) is reached with astonishing rapidity. This can be illustrated by first adding the sperm to the pH solution and then adding to the resulting sperm suspension a drop of eggs suspended in sea water. Under these conditions one would expect that the time required for the egg cortex to come to chemical equilibrium with the pH solution would be long enough to permit many more eggs to be reached by sperm and fertilized than would be the case if the eggs were allowed to come to equilibrium with the solution before adding the sperm. The results of experiments of this kind with *Asterias* eggs are given in Table II. Converse experiments were simultaneously performed; the eggs were added to the pH solution first and after 5 minutes a drop of comparatively concentrated sperm suspended in sea water was added to these eggs. When the experiment is performed as first described, the block appears at the same pH as when both eggs and sperm are at equilibrium with the pH solutions before insemination. When the experiment is reversed, the block is shifted slightly towards the alkaline side. This indicates that the essential equilibrium underlying the block involves the egg cortex rather than the sperm. The difference is hardly great

enough to be significant, though the results do show very definitely that chemical equilibrium between either eggs or sperm and the pH solutions is reached in less time than is required for sperm to reach the eggs and fertilize them.

TABLE II.

A. EFFECT OF EXPOSING *Asterias* SPERM TO pH SOLUTIONS FOR 5 MINUTES BEFORE ADDING *Asterias* EGGS IN SEA WATER. 8–31–23.

Per Cent. Eggs Fertilized.

pH									
6.2	6.6	6.8	6.9	7.0	7.1	7.2	7.6	8.15	
0	0	0	6	8	81	85	90	83	(Exp. 1)
0	0	0	0	47	85	90	89	97	(Exp. 2)

B. EFFECT OF EXPOSING *Asterias* EGGS TO pH SOLUTIONS FOR 5 MINUTES BEFORE ADDING *Asterias* SPERM IN SEA WATER. 8–31–23.

Per Cent. Eggs Fertilized.

pH								
6.2	6.6	6.8	6.9	7.0	7.1	7.2	7.6	
0	0	0	0	0	49	82	100	(Exp. 1)
0	0	0	0	2	63	95	100	(Exp. 2)

One of several experiments testing the influence of varying quantities of sperm in shifting the limits of fertilization is given in Table III. The eggs were placed in the pH solutions and 5 minutes later the sperm, diluted with the pH solutions, were added.

TABLE III.

INFLUENCE OF QUANTITY OF SPERM ON ACID BLOCK IN *Asterias*.

Per Cent. Eggs Fertilized.

pH										Quantity of sperm added to 25 cc. pH sol.
6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4	8.15	
0	0	0	50	100	95	95	100	100	100	1 cc. 1–20
0	0	0	0	35	80	95	100	100	100	1 cc. 1–200
0	0	0	0	0	20	85	87	95	90	1 cc. 1–2,000
0	0	0	0	1	40	45	25	35	30	1 cc. 1–20,000

The smallest quantity of sperm was insufficient to fertilize all the eggs even in sea water, and the largest quantity gave a dis-

tinctly opalescent suspension; yet the block did not shift beyond the limits pH 6.9 to 7.1. In general, increasing the quantity of sperm used in insemination increases the proportion of eggs fertilized in the acid solutions, but the shift to the acid side is not so great as would be expected if the failure to fertilize in the acid solutions were attributable to an impairment of the sperm. Rather the slight magnitude of this shift favors the belief that the block is due to an alteration of the properties of the egg.

It may be stated here that unless the sperm are injured or attenuated by the toxic action of egg secretions, all eggs which fertilize in the pH solutions develop normally, indicating that the fertilization reaction when once initiated in the neighborhood of the block, is completed without impairment.

The Acid Block in *Chætopterus*.

The determination of the acid block to fertilization in *Chætopterus* was made in the same manner as in *Asterias* and *Arbacia*. The egg sacks were cut in sea water and the eggs liberated from the ovaries by teasing these to pieces. The ovary fragments were removed by straining through cheese cloth, and when the eggs had maturated they were concentrated by centrifuging. A drop of the concentrated egg suspension was added to 50 cc. of the pH solution; after 5 minutes the sperm, previously diluted with the pH solutions, were added and the mixture agitated. The per cent. of fertilized eggs was determined by counting the dividing eggs one and a half to four hours after insemination.

The scarcity of material made it impossible to get more than a half dozen determinations; of these, two were discarded since only a small proportion of the eggs were fertilized in sea water. The remaining four indicated that the block appeared between pH 7.0 and 7.3, and from the two most satisfactory experiments the block was tentatively set at pH 7.1.

The acid (pH 5.8) activation of the *Chætopterus* egg, with the consequent temporary block to fertilization, has been discussed in a previous paper (3). This block, which is most effectually established by short exposures to pH 5.2 to 6.4, was tentatively ascribed to cortical changes which tend to persist after the eggs have been removed from the acid solutions, and returned to sea water. It is in no sense comparable to the physiological block occurring at

pH 7.1; the latter is perfectly and instantly reversible, as in *Asterias* and *Arbacia*, disappearing as soon as the eggs are returned to a more alkaline solution.

THE INFLUENCE OF ALKALI ON THE FERTILIZATION REACTION.

Frank Lillie (5) has observed that the addition of alkali to the sea water in which insemination occurs increases the incidence of fertilization in *Asterias* and *Arbacia* eggs of poor quality. We have confirmed this in *Asterias* and *Arbacia* and found that it is equally true for *Chætopterus*. This effect of a slight increase in alkalinity in aiding fertilization may be due to action on the sperm but it seems more probable that both the eggs and the sperm are affected. The changes which culminate in increased fluidity of the egg cortex in alkaline sea water (3) are no doubt preceded by enhanced physiological reactivity.

Apart from this stimulating action of alkali, which is not apparent in eggs of the best quality, fertilization in *Asterias* and *Arbacia* proceeds unimpaired from pH 8.15 to 9.6. With further increases in alkalinity, eggs appear in increasing numbers which have either tight or incompletely formed fertilization membranes; and at pH 10.2 the eggs have no demonstrable membranes at all. When returned to sea water after a 3 to 5 minute exposure to pH 10.0, fertilization membranes will form on most of the previously unmembraned eggs. It was concluded that these eggs were fertilized while in the alkaline solution, since the supernatant sperm carried over from the alkaline solution are incapable of fertilizing fresh eggs. Longer exposures injure the eggs to such an extent that membranes do not form on them when they are returned to sea water. The H-ion concentrations which prevent membrane elevation (and which destabilize the cortex of the unfertilized egg (4)) are shown in Figs. 1 and 2 by the dotted lines at the extreme right.

This alkaline injury is more rapid in *Asterias* than in *Arbacia*. In the latter case the eggs will divide imperfectly if returned to sea water after a 5 to 10 minute exposure to the alkaline solution. In *Asterias* the inhibition of membrane elevation is rapidly followed by a more profound injury which completely stops development. Such eggs can not be fertilized by fresh sperm in sea water.

These facts all indicate that under increased alkalinity union of the egg and sperm still occurs; but if the increased alkalinity

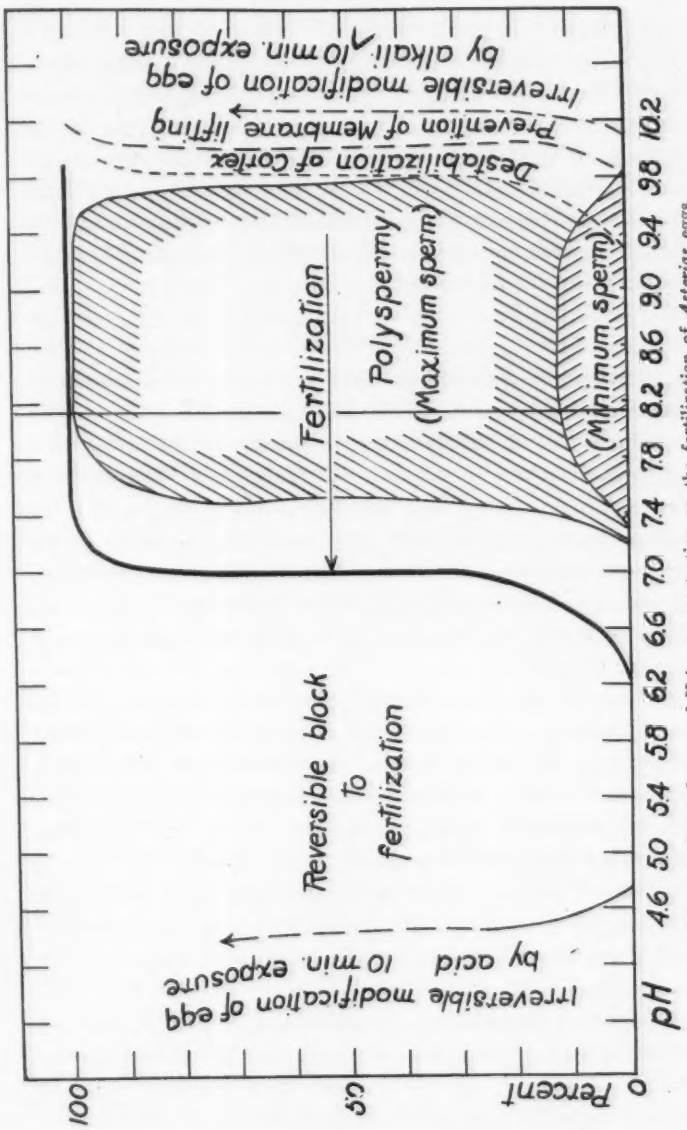

FIG. 2. The influence of H-ion concentration on the fertilization of *Asterias* eggs.

does not actually introduce some abnormality into this initial event, it impairs subsequent events of the fertilization process to such a degree as to prevent normal development. There is apparently no reversible block created by alkali corresponding to that created by acid, where the fertilization reaction proceeds in an all-or-none fashion. This conclusion is supported by the extremely rapid injury of the eggs and sperm if separately exposed to the alkaline solutions (pH 10.0), which prevent normal fertilization, and the complete absence of such injury in acid solutions (pH 6.8 to 7.0) which have a similar effect.

Polyspermy.

In the case of *Arbacia* eggs there is a very narrow range in H-ion concentration in which the incidence of polyspermy is unusually high. This range is approximately defined in Figure 1 by the heavily shaded portion; the maximum of polyspermy is close to pH 7.2. Though the incidence of polyspermy at all H-ion concentrations increases with increasing age or staleing of the eggs, yet within this narrow range, centering at pH 7.2, practically all the eggs will be polyspermic even when they are fresh and when the incidence of polyspermy is nearly zero from pH 7.4 to 9.8.

In *Asterias,* polyspermy shows no marked maximum at any H-ion concentration but occurs more or less uniformly from pH 8.5 to 9.5 (Fig. 2). When excessive quantities of sperm are used in insemination, nearly all the eggs may be polyspermic from 8.15 to 9.6. It is perhaps significant that the polyspermy curve, even though extremely broad, is limited on the alkaline side; for the incidence of polyspermy decreases appreciably before the alkalinity is sufficient to inhibit fertilization, indicating that in its general nature the underlying mechanism in *Asterias* is similar to that in *Arbacia.*

We did not have the opportunity to make similar observations on polyspermy in *Chætopterus.* Such data as we have indicate that there is, as in *Arbacia,* a comparatively narrow region in which polyspermy predominates (about pH 9.5).

Reaction of Sperm with Immature *Asterias* Eggs.

When immature *Asterias* eggs are inseminated in sea water, several sperm usually enter each egg before the fertilization membrane is formed. Subsequently the germinal vesicle breaks down and the cytoplasm acquires a mottled appearance, each sperm being the focus of a localized cytolytic process. Such prematurely fertilized eggs never attempt to divide. If sperm are added to immature eggs at various H-ion concentrations, a block appears at the same point as in the fertilization of the mature egg, viz., pH 7.0. On the alkaline side of this point the sperm enter the eggs, causing membrane elevation and the changes described above. On the acid side the sperm do not react with the eggs in any way; in the course of time, a varying proportion of these unfertilized eggs will maturate, depending on the H-ion concentration, and these, if they are returned to sea water and inseminated, will fertilize and develop normally.

Summary.

When *Arbacia, Asterias* and *Chætopterus* eggs are inseminated in CO_2-free sea water of varying H-ion concentration, a block to fertilization appears at a H-ion concentration which is constant, and apparently characteristic for each species. If the block is defined by the H-ion concentration at which 50 per cent. of the eggs fertilize, these H-ion concentrations are: *Arbacia,* pH 6.8; *Asterias,* pH 7.0; and *Chætopterus,* pH 7.1.

This block to fertilization is complete, in that eggs either fertilize and develop normally, or do not fertilize at all; and it is perfectly reversible, in that eggs which do not fertilize on the acid side of the block will fertilize immediately if they are returned to solutions on the alkaline side of the block and inseminated with fresh sperm.

In sea water more alkaline than pH 9.8 to 10.0 the fertilization process in both *Arbacia* and *Asterias* eggs is either incomplete or impaired. Apparently there is no alkaline block to fertilization corresponding in its complete reversibility to the block which appears around neutrality.

In *Arbacia* there is an increased incidence of polyspermy within a very narrow range centering at pH 7.2, indicating some critical

condition in the mechanism of fertilization at this H-ion concentration. In *Asterias* polyspermy occurs more or less uniformly over a wide range extending from pH 7.2 to 9.8.

We are indebted to Mabel T. Studebaker for the statistical work in the experiments recorded in this paper.

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HERMAPHRODITISM IN *EURYCEA BISLINEATA*.[1]

INEZ WHIPPLE WILDER AND ELIZABETH BARRETT PEABODY.

The occurrence of hermaphroditism among anurans seems to be an accepted fact. Crew ('21) summarized all the recorded cases of abnormal sexual organs in frogs and states that there are forty such cases. To this Swingle ('22) has recently added one more, but finds that of his list of forty-one abnormalties only twenty-seven can be considered hermaphrodites, a sufficient number, however, coming from the hands of so severe a critic, to warrant the statement that hermaphroditism in anurans does occur. Cerruti ('07) and King ('10) following numerous earlier writers, have investigated the occurrence of the anomaly in toads with results which, though possibly subject to differences in interpretation, tend nevertheless to substantiate the existence of hermaphroditism in these forms.

No one has done for the urodeles the service which Crew has performed for the frogs, but from the paucity of published reports upon anomalies in urodeles this would not seem to be an arduous task. Thus Chapin ('15) in reporting a case of hermaphroditism found by her in *Spelerpes bislineatus* (*Eurycea bislineata*) cited reports of only two other cases of this anomaly in urodeles which had come to her attention, one that of La Valette St. George ('95) in *Triton tæniatus*, the other that of Knappe ('86) in a young *Salamandra maculata*. Since the publication of Chapin's paper a third case has been reported by Krizenecky ('17) in *Triton cristatus*. Although the cases of La Valette St. George and of Křizenecky in *Triton* are unquestionably to be accepted as genuine, there is doubt concerning the nature of the anomaly reported by Knappe in *Salamandra*. Its interpretation as an hermaphrodite is apparently that of King ('10) who in summing up the reported cases of the occurrence of hermaphroditism in urodeles says that "Knappe ('86) noted the presence of a Bidder's organ in a young salamander." In the paper in question, however, following the enumeration of the species of Amphibia which he

[1] Contribution from the Department of Zoölogy of Smith College, No. 116.

had examined for the possible occurrence of a Bidder's organ, an enumeration which included seven species of *Anura* and two of *Urodela* (*Triton tæniatus* and *Salamandra maculata*) Knappe states definitely "Bald stellte sich heraus, dass es zur Bildung eines Bidder'schen Organs nur bei den echten Krötenarten kommt." And farther on after mentioning the unique appearance of one Bidder's organ, he says: "Eine solche Samenkörperbildung in Eikapseln des Bidder'schen Organs mit gleiches Bestimmtheit nachzuweisen, wie in dem eben beschriebenen Falle, ist mir bis jetzt nicht wieder gelungen, doch kann ich ähnliches für eine andere verwandte Thiergruppe, die Salamander, konstatiren. So liess eine in Schnittserien zerlegte Hodenabtheilung eines jungen, viellicht zweijährigen Salamandra maculata nicht den geringsten Zweifel, dass dieselbe aus Eikapseln, ähnlich denen im Bidder'schen Organ der Kröten, bestäht." It was thus obviously not Knappe's intention to state that he found a Bidder's organ in a salamander, but rather an appearance in the testis of a salamander like that of the unique Bidder's organ in a toad. In any case the interpretation of the condition described in the salamander as hermaphroditic, will depend upon the interpretation of the sexual nature of Bidder's organ itself. This is a matter which has been a bone of contention ever since the discovery of the organ in 1758 by Rösel von Rosenhof, and a number of theories have been advanced regarding its nature and significance.

In view of the almost universal agreement of modern writers as to the femaleness of Bidder's organ, Swingle's recent discussion ('21 and '22) of its nature is of great importance. In a discussion of the so-called transformation of sex in frogs, he claims that the theory is really based on a misinterpretation of the appearance of the cells in the Bidder's organ of toads. According to Swingle, the oviform-like cells of this organ do not represent the cells of an ovary, thus making the animal an hermaphrodite at this stage, but are, like the cells of similar appearance which occur in the protestis of the frogs, merely senescent male cells which are undergoing oviform degeneration. He adds further: "True hermaphroditism in frogs is a permanent and pathological condition, probably due to a mix-up in the genetic constitution of the individual,

and is not to be confused with the present problem which has to do with a normal but transitory embryological process."

The general opinion thus set forth by Swingle finds support also in a statement made by Crew ('21) who said: "Cytologically it has not been proved that the cells which constitute Bidder's organ are ovarian and there undoubtedly are reasons for questioning the generally accepted opinion that this organ is a rudimentary ovary."

The three cases of hermaphroditism already reported in urodeles, disregarding now Knappe's inconclusive report, differ from each other quite markedly. La Valette St. George's case in *Triton tæniatus* was referred to by Cole ('96) as "the most complete case of hermaphroditism yet recorded among the Amphibia." The specimen was a male with perfectly distinct and independent paired ovaries, in addition to a pair of normal testes, but, however, without any traces of oviducts. The testes contained developing and fully developed sperms; and the ovaries, eggs in various stages of maturity.

The case reported by Křizenecky in *Triton cristatus* showed the presence of ova within both peripheral and internal lobules of otherwise normal testes.

The case reported by Chapin in *Eurycea bislineata* was that of an advanced larva in which the gonad was essentially male with female elements. Macroscopically, the anterior part of the left gonad, which was much reduced in size, resembled the normal testis in texture, though not in shape, while the posterior region was distinctly like an ovary. The right gonad, which was somewhat smaller than the normal testis of an individual of the same size, showed another sort of hermaphroditism. Two ova were found in the otherwise apparently normal testis, each one completely filling one lobule, which would normally contain a large number of male cells. This case showed, therefore, two ways in which female elements may be disposed in otherwise distinctly male gonads; one in the form of growing ova among the cysts of spermatogonia, and the other by a modification of a part of the gonad into a region resembling an ovary. The numerous cases of hermaphroditism which we have found in this same species are all of the same general character as that described by Chapin.

In the light of a recent article by Jordan ('22), it may be well to define our use of the term, hermaphroditism. According to Jordan, true anatomic hermaphroditism occurs " where ovary and testis are present in the same individual." Jordan regards the presence of an ovo-testis as a modification of true hermaphroditism, a condition which he designates as a type of false hermaphro-

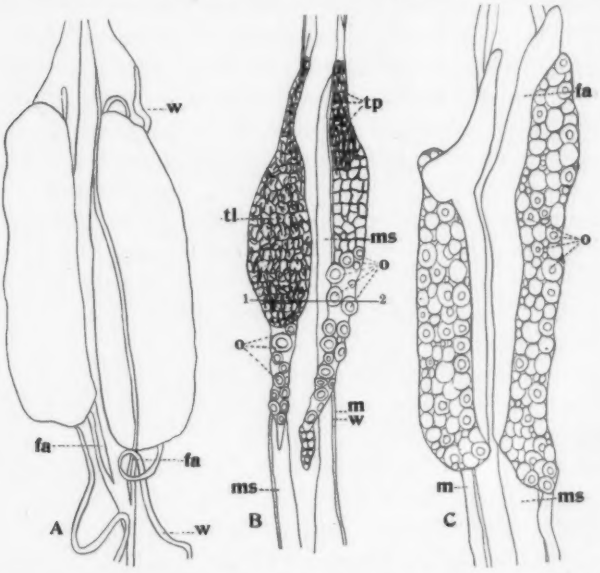

FIG. 1. Camera lucida drawings of the ventral view of the gonads of (*A*) an adult male; (*B*) an adult hermaphrodite; and (*C*) an adult female (× 9). *Fa*, fat bodies; *m*, Müllerian duct; *ms*, mesonephros; *o*, ova (primary oöcytes). *tl*, testicular lobules; *tp*, testicular pigmentation (the two latter present but not shown in (*A*)); *w*, Wolffian duct. The line 1–2 shows the level of the section drawn in Fig. 4.

ditism. Were this distinction to be accepted, the term, true hermaphroditism, could be used only when referring to such a case as that of La Valette St. George's in *Triton tæniatus*. There seems to be no justification, however, for this distinction of Jordan's, inasmuch as a distinct testis and ovary is but a further step in the separation of the male and female elements which, in some individuals, are still intermingled to a greater or less extent in the ovo-testis. An examination of the adult ovo-testis shown in

Fig. 1 *B* shows that a separation posterior to the testicular part on each side, such as, in fact, is slightly indicated in the right gonad, would transform each ovo-testis into a distinct testis and ovary. A female was found in which the ovary (Fig. 2) showed a number of separate parts or lobes, some connected with each other by

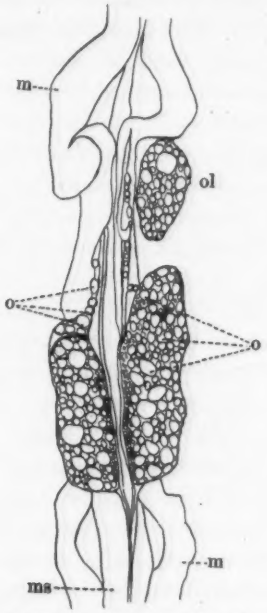

FIG. 2. Camera lucida drawing of the ovaries of an adult female showing an unusual lobed form (× 7). *M*, Müllerian duct; *ms*, mesonephros; *o*, ova; *ol*, detached lobe of ovary.

the mesovarium and others quite distinct, serving to illustrate the point that parts which are usually continuous may, through some unknown cause, become thus carried apart. Jordan's distinction in terminology seems, therefore, a somewhat arbitrary one, at least as applied to our species, and thus any individual which shows the presence of both male and female sex cells, even though these appear side by side in the same gonad, is regarded in this paper as a true hermaphrodite.

Naturally the ultimate criterion of hermaphroditism should be the production of functional germ cells of both sexes. Such a

criterion would obviate all possibility of the condition being given the interpretation which Swingle has given to the oviform cells which occur in Bidder's organ and in the larval testis (pro-testis) of frogs. In carrying out our investigation we had in mind as near an approach to this ideal as possible, and, having found examples of the condition which to us seemed unquestionably hermaphroditic in individuals of various stages up to transformation, we made a definite search for such cases among adult animals. As this search was rewarded by the discovery of one adult in which the hermaphroditic condition was beyond question, although the individual had not arrived at full sexual maturity, we feel confident that our interpretation of our cases as true hermaphrodites is correct and that the condition described cannot be considered as "a normal but transitory embryological process."

Percentage of Occurrence.

The determination of the percentage of occurrence of hermaphrodites with reference to that of males and females in *Eurycea bislineata* is based upon the examination of the gonads of 1113 individuals ranging from the typical larval to the adult stage. Wilder ('24) has shown that *Eurycea bislineata* is a form in which the period before transformation is considerably prolonged, covering from two to three years, although the structural changes leading toward metamorphosis are inaugurated many months previous to the actual transformation. The whole period from hatching to transformation is subdivided on the basis of structural changes into stages, the readily recognizable criteria of which, in living individuals, are as follows:

1. Postembryonic stage—Yolk still present, intestine not fully formed.
2. Typical larval stage—Intestine fully formed; no naso-lacrimal groove and no *os thyreoideum*.
3. Premetamorphic stage—Open naso-lacrimal groove (in incipient phase); *os thyreoideum* present; no vesicular glands in the skin.
4. Metamorphic stage—Glands of skin appearing as tiny acinous vesicles (in incipient phase), becoming rapidly larger and more conspicuous; absorption of larval structures and de-

velopment of eyelids and naso-labial groove (advanced phase).

The specimens used constituted representative collections made through a period of several years and had been preserved either in alcohol after fixation in Bouin's solution, or in formalin.

The method followed in sexing was first to examine the gonads *in situ* under a Bausch and Lomb binocular dissecting microscope with a strong artificial illumination. In many cases this was sufficient to diagnose the sex, but in those cases in which it was not, the gonad was removed and cleared *in toto* in glycerine for more careful study under the compound microscope. If in sexing one begins with adults and continues through the smaller and earlier stages, one comes finally to a point where it is practically impossible to be sure of the sex. Individuals of less than 27 mm., though frequently possessing readily sexed gonads, more often exhibit a developmental condition which might admit of various interpretations, since, at least without the use of cytological criteria, the small cells present might be either oögonia or spermatogonia. Bouin ('01) found that in *Rana temporaria* the first development of male and female germ-cells is identical as far as origin and general appearance are concerned. It may even be the case in *Eurycea,* as Okkelberg ('21) has shown for the brook lamprey, that the animal passes through a period of sex indifference before sex differentiation sets in. His observations "seem to warrant the conclusion that each larva of this species (*Entosphenus wilderi*) carries the potentiality of both sexes and that sex, therefore, is not irrevocably fixed at fertilization." He explains the development of sex in these gonads of "potentially either" sex by showing the presence in the gland of two kinds of germ cells, those manifesting a tendency towards rapid division (katabolic) and those showing a tendency to growth (anabolic). He says: "The former are regarded as having a male and the latter a female potentiality. The relative proportion of anabolic and katabolic cells determines whether the larva becomes a male or a female individual."

Since our problem, however, was not one dealing with a possible early transitory hermaphroditic condition, we have included in the calculation of the percentage of occurrence only those indi-

viduals which had definitely passed beyond the indeterminate sexual stage to a point where sex could be definitely diagnosed. The following summary is, therefore, based upon the study of gonads of animals of a length of 27 mm. and over, the arbitrary minimum of 27 mm. being taken as approximately representing the dividing line between those individuals in which the sex is still, if not indifferent, at least frequently difficult to determine, and those in which the sex is unquestionably established and recognizable.

SUMMARY OF PERCENTAGE OF OCCURRENCE.

Typical larval individuals (of 27 mm. and over in length)

Total number sexed	178	
Number of males	86,	or 48.3 %
Number of females	89,	or 50.0 %
Number of hermaphrodites	3,	or 1.68%

Individuals in incipient phase of premetamorphosis

Total number sexed	333	
Number of males	177,	or 53.2 %
Number of females	152,	or 45.7 %
Number of hermaphrodites	4,	or 1.2 %

Premetamorphic individuals (beyond incipient phase)

Total number sexed	256	
Number of males	119,	or 46.5 %
Number of females	134,	or 52.3 %
Number of hermaphrodites	3,	or 1.2 %

Metamorphic individuals

Total number sexed	226	
Number of males	115,	or 50.8 %
Number of females	107,	or 47.4 %
Number of hermaphrodites	4,	or 1.8 %

Adult individuals

Total number sexed	120	
Number of males	77,	or 64.0 %
Number of females	42,	or 35.0 %
Number of hermaphrodites	1,	or 0.83%

All individuals exclusive of adults

Total number sexed	993	
Number of males	497,	or 50.05%
Number of females	482,	or 48.53%
Number of hermaphrodites	14,	or 1.41%

All individuals

Total number sexed	1113	
Number of males	574,	or 51.57%
Number of females	524,	or 47.08%
Number of hermaphrodites	15,	or 1.35%

The variation in the percentage of occurrence of hermaphrodites in the different developmental stages has little significance because of the large probable error due to the small number of specimens examined. It should be noted that the nearest approximation to the general average, 1.35 per cent., occurs in the case of those stages in which there were the largest numbers examined. The extremely low percentage in the adult group is noteworthy and, though probably due to the small number of specimens used, may conceivably indicate a lower degree of viability in the case of hermaphrodites in adult life. The discrepancy in percentage of males and females in the adult group may also indicate a difference in viability, but is more likely to be due to the fact that the collections used were made mainly in the spring when the females would be more difficult to find because during the egg laying period they are under large rocks in the deeper water.

The fact of real significance is that of the existence of hermaphroditism in every developmental stage, since together with the approximate equality of the two sexes, it serves to eliminate any claim that the condition in question is, in this species, merely a transitory one.

This establishment of a fairly constant percentage of occurrence of hermaphroditism in *Eurycea bislineata* suggests the possibility that a search for the phenomenon in other urodeles might reveal a like frequency of occurrence. The urodeles offer an inviting field for such investigation since so little has been done with them in connection with the problem in contrast to the large amount of attention which has been given to the anurans.

Description of Representative Cases of Hermaphroditism in *Eurycea bislineata*.

Adult Stage.

The one hermaphroditic adult found was an individual of 65 mm. in length which had the external characteristics of a female,

as shown by the presence of a spermatheca in the dorsal wall of the cloaca, and the shape of the head. The appearance of the gonads *in situ* is shown in Fig. 1 *B*. For comparison, a typical ovary and a typical testis from animals of approximately the same size and collected at the same time (July 3, 1915) are shown in Figs. 1 *A* and 1 *C*. The smaller size of both testicular and ovarian parts of the ovo-testis as compared with the typical male and female gonad respectively will be noted. Moreover, while the reproductive ducts (Wolffian and Müllerian respectively) of the normal male and female have approximately the form characteristic of

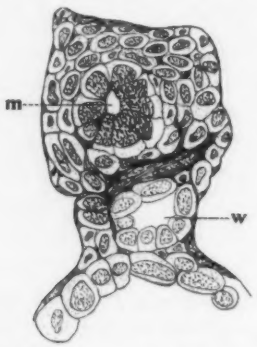

FIG. 3. Cross section showing (*m*) the Müllerian and (*w*) the Wolffian ducts in the adult hermaphrodite (cf. Fig. 1 *B*) (× 400).

the mature condition, the hermaphrodite showed macroscopically apparently a single slender straight duct upon each side following the lateral border of the mesonephros. Subsequent microscopic examinations of cross sections of this, however, revealed the presence of two ducts (cf. Fig. 3) the more lateral of which, the Müllerian, alone persists anterior to the mesonephros. This sexually indifferent condition of the ducts is identical with that shown by cross sections, made previous to this investigation, through the body of a 66 mm. immature adult male, the testes of which also correspond histologically almost exactly to the testicular portions of the hermaphroditic gonad and were thus used as a typical male control in the microscopic study.

The fat bodies in the hermaphrodite were especially large, and when the body cavity was first opened completely obscured every-

thing beneath them, making their removal necessary for the study of the gonads. The right gonad is the larger and in a macroscopic examination seems to be primarily a testis with characteristic pigmentation and conspicuous lobules. The pigmentation is, however, somewhat lighter in color than that usually found in the adult testis. The length of the testicular portion of the right gonad is 4 mm., while that of the testis shown in Fig. 1 *A* is 5.25 mm. Posterior to this testicular portion, occurs a more slender unpigmented structure in which ten large unmistakable ova, together with smaller ones, may be seen. Its general resemblance to an ovary is seen by comparison with the ovaries of the 60 mm. adult female shown in Fig. 1 *C*.

The left gonad is longer and more slender as a whole than the right. This is due to the greater length of the ovarian part, the testicular region being smaller than that of the right gonad (2.75 mm. as compared with 4 mm.). Moreover the testicular pigmentation is confined to the anterior region of the gonad and is still lighter in color than that of the right gonad. The characteristic lobules are present, but there is less differentiation of the testicular from the ovarian region, the two seeming to grade into each other insensibly. In this gonad 14 large ova are in evidence as well as numerous smaller ones. At the extreme posterior end of the left gonad there is a small semi-detached ovarian lobe.

The hermaphrodite had not been preserved originally for histological study, since the animal had been killed in 5 per cent. formalin and had been kept in this fluid since 1915. Nevertheless the gonads were sectioned, and, in spite of the excessive shrinking which is especially evident in the separation of the cysts which make up the testicular lobules, the characteristic structure of both the male and the female components was shown with unmistakable clearness.

Figure 4 shows a cross section through a region where, in a macroscopic examination, the right gonad had the appearance of a testis and the left one the appearance of an ovary. In general this section shows the typical testicular structure of the right gonad, with lobules, each made up of a number of component cysts of male cells, arranged radially about a central collecting duct. A single large ovum appears in the section, however, completely fill-

ing one of the lobules and thus apparently the equivalent of many cysts. The characteristic anabolic and katabolic nature of the female and male cells respectively is thus well exemplified. The female cell grows, the male cell divides. In the whole series of sec-

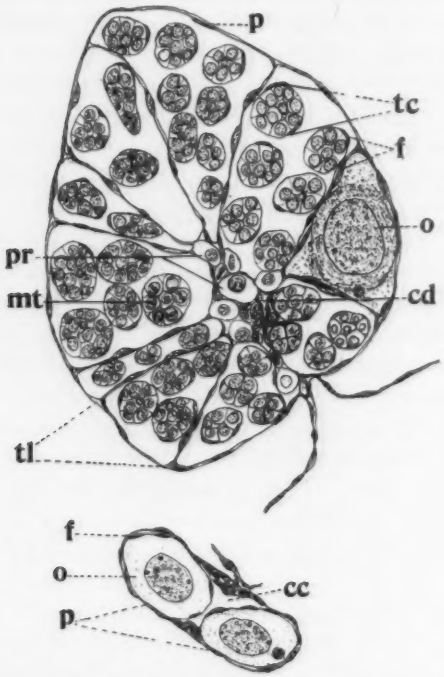

FIG. 4. Cross section through the gonads of the adult hermaphrodite at the level indicated in Fig. 1 *B* by the line 1–2 (× 87). The two gonads are brought nearer together in the drawing than their actual position. *Cc*, central cavity of ovarian region; *cd*, collecting duct of testicular region; *f*, follicles of both ova and testicular cysts; *mt*, spermatogonial cells in mitosis; *o*, ova (primary oöcytes); *p*, peritoneal investment; *pr*, primordial germ cells; *tc*, testicular cyst; *tl*, testicular lobule.

tions through the gonads no fewer than ten such ova were found in the testicular portion of the right gonad and six in that of the left. All were, like the one shown in Fig. 4, in an apparently normal state of development, manifesting no incipient signs of degeneration such as Crew ('21) reports to be the case in Anura whenever

female elements are found in parts which are primarily male in character. In fact no difference could be detected between the ova among the testicular lobules and those of the more distinctly ovarian part of the gonads except that the former had advanced further in the matter of accumulation of layers of yolk.

There was much mitotic activity in progress in the testicular lobules, the same stage of mitosis being exhibited by all the cells of a given cyst, a condition which is to be expected if one postulates their formation by repeated divisions of a single primordial spermatogonium. Thus the male elements, like the female, have every appearance of undergoing perfectly normal development. In the transition region from the testicular to the ovarian part of the gonad, small testicular lobules appear which are somewhat degenerate in character.

The posterior part of each gonad shows the typical ovarian structure as demonstrated by the section of the left gonad in Fig. 4, with large central cavity surrounded by ova, each within its layer of follicle cells.

The microscopic condition thus shown seems scarcely more advanced than that pictured and described by Chapin ('15) in the gonads of her 46 mm. hermaphroditic "larva," which, in the absence of the more exact criteria of developmental stages of the whole larval life such as we are here making use of, was designated as a "larva" in the sense that it had not yet undergone transformation. In reality it was probably an individual which was approaching metamorphosis if not in actual metamorphic condition. In general this species shows much normal variation in the developmental condition of the gonads at transformation and it is thus not surprising that one individual previous to transformation should be in the same condition as another which has already transformed.

Our adult hermaphrodite is noteworthy, not only because it shows that the condition is not merely a juvenile one, but also because so far as external characters are concerned it appears to be a female. These characters, it must be confessed, are not of a very decided nature in this species, the presence of a spermatheca being, indeed, the only unquestionable one. Moreover, the cloacal papillæ which are the characteristic male structures, might

not have appeared in so immature an individual and thus one cannot be sure that later the cloaca might not have shown male as well as female structures.

Crew ('21) in his summary of the recorded cases of abnormality of the reproductive system, says that of the 30 frogs of which sufficient details were given as to their secondary sexual characters, 25 (83.3 per cent.) were definitely and typically males; 4 others were definitely but imperfectly male (13.3 per cent.); and in the remaining case, a *Rana temporaria* described by Huxley ('20), the secondary sexual characters were female (3.3 per cent.). He says: "The abnormalities which have been recorded can be so tabulated that the first case most nearly approximates to the normal female and the last the typical male, with respect to the nature of both primary and secondary sexual characters. Thus arranged it is seen that the cases furnish an almost complete series of gradations which range from an individual almost completely female, to one almost completely male, and that the conditions found readily appear to be merely graded stages of a single process."

All of our other hermaphroditic examples of *Eurycea* were in too early a stage of development for secondary sexual characters to have appeared. However, so far as the condition of the gonads alone was concerned the same sort of graded series was found as that described by Crew in the frogs.

More thorough microscopic examination of gonads might, by disclosing occasional ova among the lobules of an otherwise normal testis or a few testicular elements concealed by the large ova of an ovary, yield a more complete seriation. At least the conditions shown by *Eurycea* indicate that in this species the hermaphroditic condition cannot be interpreted as always a modification of the same sex.

Metamorphic Stage.

In the more advanced developmental stages, as in the case of the adults, the sexing of the specimens consisted in distinguishing between a large unpigmented ovary full of bulging ova and a more slender, heavily pigmented testis with, of course, attention directed toward the detection of any combination of the two, which would mean an hermaphroditic condition. Fig. 5 *B* shows the hermaph-

roditic gonads of a 42.4 mm. animal in the advanced metamorphic stage. The gonads are essentially male so far as general shape and slight characteristic pigmentation are concerned, and there are visible in them ten unmistakable ova. Figs. 5 *A* and 5 *C* represent the gonads of a typical male and female collected on the

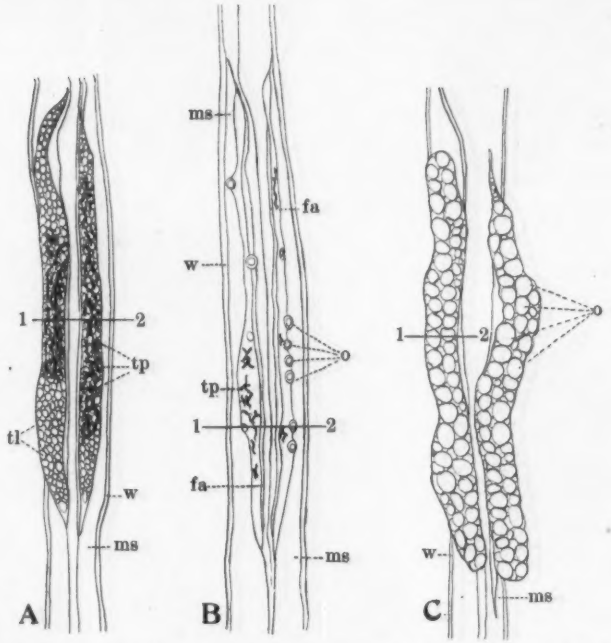

FIG. 5. Camera lucida drawings of the ventral view of gonads of (*A*) an incipient metamorphic male, length 40.9 mm.; (*B*) an advanced metamorphic hermaphrodite, length 42.4 mm.; and (*C*) a premetamorphic female, length 40.2 mm. (× 15). *Fa*, fat bodies; *ms*, mesonephros; *o*, ova; *tl*, testicular lobules; *tp*, testicular pigment; *w*, Wolffian duct. The levels of the sections of the gonads shown in Fig. 6 are indicated by the lines 1–2.

same date and of approximately the same size and stage of development, which may be used for comparison. As noted before, the smaller size of the hermaphroditic gonad, and of the ova present in it, is obvious.

Cross sections 10 micra thick were made through all three pairs of gonads and were stained, some with Delafield hæmatoxylin,

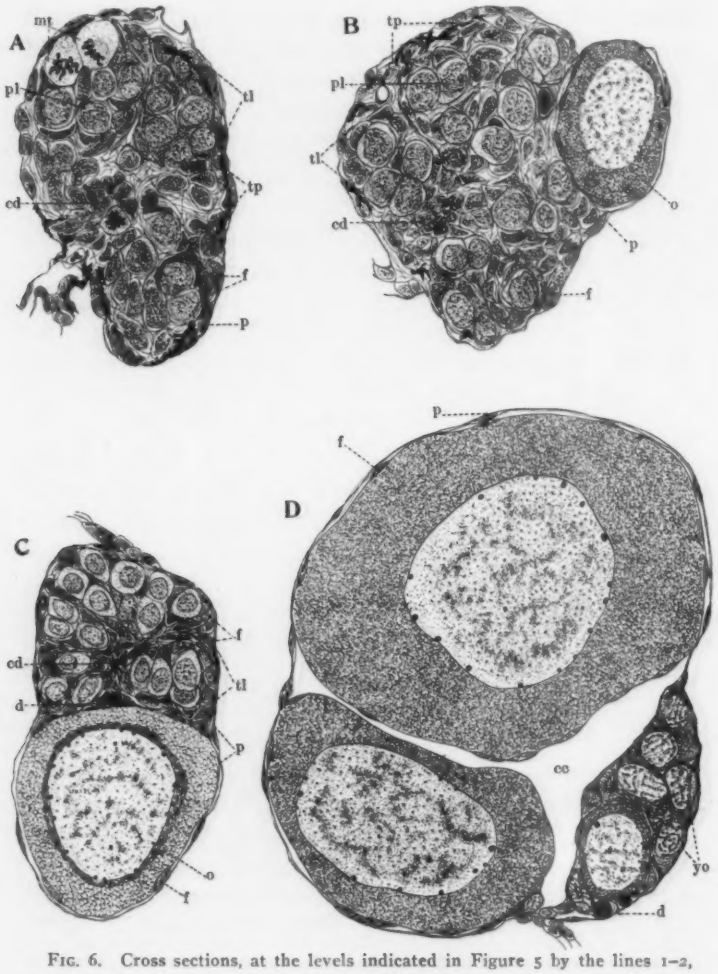

FIG. 6. Cross sections, at the levels indicated in Figure 5 by the lines 1–2, showing (*A*) the right gonad of an incipient metamorphic male; (*B*) the right gonad of an advanced metamorphic hermaphrodite; (*C*) the left gonad of the same individual; and (*D*) the right gonad of a premetamorphic female (× 365). *Cc*, central cavity of ovary; *cd*, collecting duct of the testis and testicular region of the hermaphroditic gonad; *d*, degenerating cells; *f*, follicles; *mt*, spermatogonial mitoses; *o*, ova (primary oöcytes); *p*, peritoneal investment; *pl*, polymorphonuclear germ cells (primary spermatogonia); *tl*, testicular lobule; *tp*, testicular pigmentation; *yo*, young primary oöcytes.

others with iron hæmatoxylin, and still others with safranin and light green. The lobules of the normal testis (Fig. 6 *A*) are made up of spermatogonia surrounded singly or in small groups with follicle cells, with which the cysts later to be formed by the division of these spermatogonia will be covered. Typical spermatogonial mitoses are seen in this and in other sections. In the hermaphroditic gonads (Figs. 6 *B* and *C*) we find a testicular structure corresponding in general to that shown by the normal testis, with typical mitoses in evidence. At the level shown in *C* in which the ovum constitutes practically half of the total diameter of the gonad, the testicular part is not quite so far advanced as in *B*, but is, in fact, in much the same condition as the more anterior region of the normal testis. The ova shown in both of these sections are typical, as will be seen by comparison with the section of the normal ovary (Fig. 6 *D*), although they are not equal in size to the larger ones of the normal ovary.

Incipient Premetamorphic Stage.

In the examination of younger stages in which little or no testicular pigment had developed, reliance for the diagnosis of the sex had to be based upon the shape of the ovary with its protruding ova to distinguish that organ from the slender testis or from the testis with female elements present in it.

Figure 7 *B* shows the general appearance of the hermaphroditic gonad of a 36 mm. incipient premetamorphic individual, and Figs. 7 *A* and *C* show gonads of a typical male and female of about the same size and developmental condition. The smaller size of the hermaphroditic gonad is again evidenced. The ova are of about the size of the smallest seen in the normal ovary.

The anterior part of the reproductive organs in each case was sectioned transversely for the purpose of studying the relation of the ducts; while the posterior part, including, in fact, the major part of the gonads themselves, was sectioned horizontally. Delafield hæmatoxylin and iron hæmatoxylin staining were used.

Histologically the developmental condition of these gonads as shown in Fig. 8 *A*, *B*, and *C* is not essentially different from that of the metamorphic stage, except that both in the normal testis and in the testicular region of the hermaphroditic gonad there are

more single spermatogonia and fewer in groups, although the lobulated structure of the gonad is evident. There were fewer instances of spermatogonial mitosis, none, in fact, discovered in the hermaphrodite, the sections of which, however, were somewhat

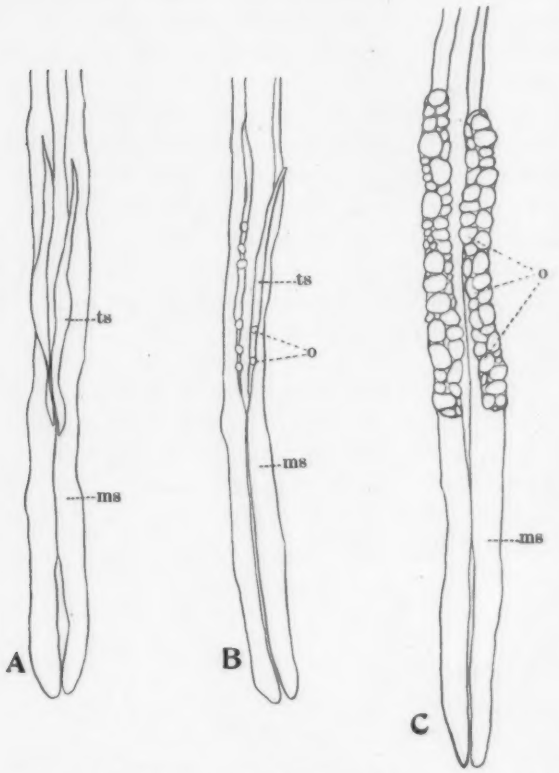

FIG. 7. Camera lucida drawings of the ventral view of the gonads of (*A*) an incipient premetamorphic male, length 37.4 mm.; (*B*) an incipient premetamorphic hermaphrodite, length 36 mm.; and (*C*) an incipient premetamorphic female, length 36.2 mm. (× 15). *Ms*, mesonephros. *o*, ova; *ts*, testis and testicular portion of hermaphroditic gonad.

fragmentary. In every other particular of cell arangement and nuclear structure the testicular regions of the hermaphrodite were identical with the normal testis. The ova of the hermaphrodite,

though of smaller size, were perfectly normal in appearance. They have the typical relationship to the testicular lobules, and, owing to their earlier stage of growth, do not bulge out so conspicuously from the surface of the gonad as in the case of the metamorphic stage (cf. Fig. 6 *B* and *C*).

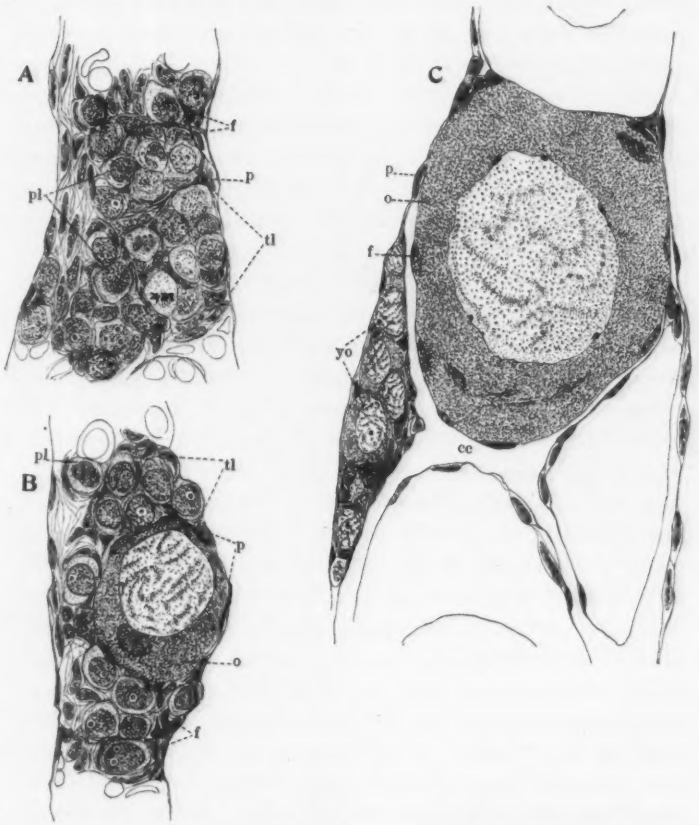

FIG. 8. Horizontal sections through corresponding regions of the gonads shown in Fig. 7 of (*A*) male; (*B*) hermaphrodite; (*C*) female (× 356). *Cc*, central cavity; *f*, follicles; *o*, ova (primary oöcytes); *p*, peritoneal investment; *pl*, polymorphonuclear germ cells (primary spermatogonia); *tl*, testicular lobule; *yo*, young primary oöcytes.

Typical Larval Stage.

With the younger stages, macroscopic evidence could be relied upon still less for sexing, although normal ovaries are easily recognizable if the growth period of the ova has been well entered upon, and such normal female gonads packed with growing ova of approximately uniform size have been distinguished macroscopically in larvæ as small as 25 mm. in length, though our percentage data (p. 8) did not include individuals under 27 mm. in length. The difficulty in sexing lies in the uncertainty as to the presence of male elements in gonads in which the ova are few in number but unmistakable. We have not as yet examined microscopically large numbers of gonads of young larvæ. However, in looking over our laboratory sets of serial sections of larvæ collected in September or early October, ranging in length from 17 to 25 mm. and presumably about 12 weeks old, we find that while a few of them show a condition which might be considered as sexually indifferent in that the gonads are made up of typical primordial germ cells, each with its investment of follicle cells, arranged in single rank about a central cord, a larger number of those examined, including some of the smallest individuals, show practically all of the germ cells in early maturation stages (leptonene and pachytene) or as growing oöcytes. Such an individual seems to us to be a female, since other individuals show gonads made up of more numerous, smaller germ cells grouped in such a manner as to suggest at once the incipient lobules of a typical testis. In such gonads the germ cells show no maturation phenomena, although mitosis is ocasionally seen. For the most part the nuclei are either polymorphic, or in rounded form with one or more conspicuous nucleoli. As we have very few data as to the condition of the gonads the following spring, we can only express here our tentative opinion that this species, in spite of its prolonged larval life, exhibits no such early larval maturation of male germ cells synchronously with that of the female germ cells, as has been described by Swingle ('21) for *Rana catesbeiana*.

To push our power of diagnosis of sex and recognition of possible hermaphrodites back into these early stages demands as a basis, not only a careful and thorough cytological investigation of the origin and differentiation of the germ cells such as that of

Bouin ('01) and Dustin ('07) for other species of Amphibia (mainly *Anura*), and Okkelberg ('21) for the brook lamprey, but also a complete bridging over of the gap between the early developmental phenomena and the seasonal sexual phenomena of adult life.

With regard to the bearing which occasional hermaphroditism such as this has upon its regular occurrence in certain species of animals, and upon the significance of the phenomenon in general, two opposing views are held. One of these, as set forth by Doncaster ('14), regards hermaphroditism not as a primitive but as a purely secondary condition. This opinion is based mainly on the fact that the hermaphroditic species of animals are, for the most part, highly specialized ones. Sporadic hermaphroditism is thus considered an example of variation along this same direction.

The other view is that which has recently found so vigorous a supporter in Jordan ('22), that hermaphroditism, at least in the vertebrate group, is a primitive character. Jordan points out "the abundant evidence of a normal hermaphroditic condition either adult or juvenile, among lower vertebrates (*e.g.*, tunicates, cyclostomes, probably some Amphibia)," and that "the early gonads with their primordial germ cells appear identical." This view of the primitive character of hermaphroditism naturally goes hand in hand with the theory that sex determination is a matter of differential metabolism and that forms in which sex determination has become bound up in the chromosomes represent a higher stage in metabolic control of the developing organism.

Jordan points out the peculiar interest presented by the case of amphibians in this connection, since most investigators have failed to find any evidence of a sex chromosome in this group, although King ('12) describes it for *Necturus maculatus*, Levy ('15) for *Rana esculenta*, and Swingle ('17) for *Rana pipiens*. In a later paper, however, Swingle ('21) questions the correctness of his own earlier identification of an accessory chromosome in *Rana pipiens* and suggests the strong probability that Levy may also have been mistaken.

Jordan makes the suggestion that the Amphibia may constitute a group in which the evolution of the sex chromosome as a separate element can be traced, and in which also a general ten-

dency toward juvenile hermaphroditism bridges the gap between lower vertebrates where functional hermaphroditism occurs in certain classes and higher vertebrates where the condition occurs only as an anomaly. Swingle has done much to dispel the idea of juvenile hermaphroditism in the anurans. Cases of hermaphroditism such as we have here described in *Eurycea bislineata* give every evidence of being a permanent rather than juvenile condition. On the other hand, although we have thus far found no evidence that these permanent hermaphrodites arise out of an earlier condition in which the gonads have the potentiality for both sexes and may thus be regarded as capable of producing either males, females, or hermaphrodites, we do not feel that our investigation of these early stages has been sufficiently extensive to warrant us in excluding this possibility. In the absence of evidence of a chromosomal control of sex determination in this species, and indeed in Amphibia in general, one should maintain an open mind toward other possibilities. Much further investigation of the subject is therefore obviously needed and is now in progress.

SUMMARY.

1. True hermaphroditism occurs in approximately constant proportions in every developmental stage of *Eurycea bislineata* from typical larval to adult.

2. The percentage of occurrence of hermaphrodites in this species, based upon the examination of 1,113 individuals, is 1.35 per cent.

3. There are now on record 18 cases of hermaphroditism in urodeles. The first is that of La Valette St. George ('95) in *Triton tæniatus,* the second is that discovered by Chapin ('15) in *Eurycea bislineata,* the third is that of Křizenecky ('17) in *Triton cristatus,* and the other 15 cases, in *Eurycea bislineata,* are presented in this paper.

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November 13, 1923.

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